Characterization of Microsomal Choloyl-Coenzyme A Synthetase

By DONALD A. VESSEY* and DAVID ZAKIM†

Liver Studies Unit, Molecular Biology Division† and Department of Medicine† Veterans Administration Hospital, San Francisco, CA 94121, U.S.A., and Departments of Medicine*† and Biochemistry* University of California, San Francisco, CA 94143, U.S.A.*

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Choloyl-CoA synthetase (EC 6.2.1.7) was characterized for the first time under appropriate assay conditions. The pH optimum for the reaction is pH 7.2–7.3. The reaction has an absolute requirement for bivalent cation. Several different metal ions fulfil this requirement, but Mn^{2+} and Mg^{2+} were the most effective. The K_m^{app} (apparent K_m) for CoA, extrapolated from kinetic data, is $50 \mu M$, but in fact the rate of reaction is increased little by concentrations of CoA above $25 \mu M$. The K_m^{app} for ATP is $600 \mu M$. High concentrations of ATP appear to cause substrate inhibition. The K_m^{app} for cholate was $6 \mu M$. The enzyme was inhibited by treating the microsomal fraction with *N*-ethylmaleimide. The inclusion of various conjugated and unconjugated bile salts in the assay also inhibited the enzyme. Unconjugated bile salts were more potent inhibitors than the conjugated bile salts. High concentrations of oleic acid inhibited the enzyme. The properties of choloyl-CoA synthetase were not modified by alterations of the properties of the lipid phase of the microsomal membrane. Treatment with phospholipase A did not alter activity directly. Triton N-101 and Triton X-100 also were without effect on activity, and the enzyme was insensitive to temperature-induced phase transitions within the lipid portion of the membrane. The enzyme can be solubilized from the microsomal membrane in an active form by treatment with Triton N-101.

The liver produces two primary types of bile acids, cholic acid and chenodeoxycholic acid, as the final metabolic products in the catabolism of cholesterol. Large amounts of these compounds (and also secondary bile acids, which are produced by bacterial action within the lumen of the intestine) are found in mammalian bile in the form of glycine or taurine conjugates. Conjugation of bile acids appears to increase the rate at which they are secreted into the bile (O'Maille *et al.*, 1965). In addition, conjugated as compared with unconjugated bile acids are more effective promoters of absorption within the intestine because they are less readily reabsorbed from the lumen of the intestine by passive diffusion (Schiff *et al.*, 1972). Conjugated bile acids also appear to be less toxic to the intestinal mucosa. For example, 8 mM-cholate caused a 99% inhibition of glucose transport by segments of jejunum, whereas equivalent concentrations of glycocholate or taurocholate were without effect (Pope *et al.*, 1966). This correlates with the finding that elevated amounts of free bile acid in the intestine produce glucose malabsorption in infants (Rodriguez *et al.*, 1974). Also cholate, but not taurocholate, leads to nearly complete inhibition of esterification of palmitate in the intestine (Clark *et al.*, 1969). Deoxycholate is known to produce gross morphological damage to the jejunum, in con-

trast with conjugated deoxycholate (Teem & Phillips, 1972), which correlates with the greater sensitivity of jejunal water absorption to inhibition by deoxycholate as compared with conjugated deoxycholate in hamsters (Teem & Phillips, 1972) and rats (Harries & Sladen, 1972). Conjugation thus seems to serve important physiological functions.

The enzymes that catalyse the conjugation of bile acids have not been studied carefully, despite their biological importance. However, conjugation of bile acids with glycine or taurine is known to involve a two-step process (Bremer, 1956; Elliott, 1955; Siperstein & Murray, 1955). The bile acids are converted into a CoA derivative in the initial reaction (reaction 1). The bile acid acyl-CoA compound reacts with glycine or taurine to produce the conjugated bile acid (reactions 2a and 2b).

It is not known whether a single enzyme catalyses the conversion of all bile acids into their CoA

derivatives, or whether there are multiple substrate-specific forms for this step. Similarly, the number of enzymes catalysing reactions 2(a) and 2(b) is unclear. There has been a previous attempt to characterize the enzyme catalysing reaction (1) (Elliott, 1956), but as demonstrated below, inappropriate assay conditions and the failure to determine initial rates of reaction yielded artifacts. The purpose of the experiments reported in the present paper is to begin to clarify these matters.

In this paper we have studied the properties of the enzyme catalysing the synthesis of choloyl-CoA [cholate-CoA ligase (AMP-forming), EC 6.2.1.7]. Since this enzyme is located in the microsomal fraction of the liver cell, we have chosen to characterize the enzyme *in situ* rather than attempting initially to isolate it from its membrane milieu. The basis for this decision is the rapidly expanding evidence that the properties of many membrane-bound enzymes depend on interactions between the enzyme and its environment within the membrane (cf. Zakim & Vessey, 1975). Since we are interested in delineating the properties of the native, or untreated, form of choloyl-CoA synthetase, we considered it important to characterize the enzyme at first as it occurs in its microsomal environment. Only in this way can one interpret results of subsequent attempts at purification.

Experimental

Materials

[carboxy- ^{14}C]Cholic acid was obtained from New England Nuclear, Boston, MA, U.S.A. The specific radioactivity was decreased to 28 mCi/mmol before use. Triton N-101 was obtained from Sigma, St. Louis, MO, U.S.A., as was CoA (lithium) which contained 95% reduced CoA. Bio-Gel A5m (100–200 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. The microsomal fraction of guinea-pig liver was isolated in 0.25 M-sucrose as described previously (Zakim & Vessey, 1973). The activity of choloyl-CoA synthetase was stable for at least 1 month when the microsomal fraction was stored at -50°C .

Methods

Choloyl-CoA synthetase activity was measured by a radiochemical assay. The standard assay mixture contained 16 μM -[^{14}C]cholate, 100 μM -CoA, 5 mM- Mg^{2+} , 5 mM-ATP, 0.8 mg of microsomal protein/ml and 100 mM-Tris/HCl, pH 7.3, in an assay volume of 0.1 ml. The reactions were started by the addition of either microsomal fraction or CoA and stopped by the addition of 0.5 ml of butan-1-ol that was saturated with water and 0.5 ml of 5 mM-EDTA. A two-phase system was produced, and the phases were

separated by centrifugation. The aqueous phase contained [^{14}C]choloyl-CoA, and the unchanged [^{14}C]cholate was extracted into the butanol layer. The aqueous layer was removed, washed once with 0.5 ml of butan-1-ol saturated with water, and then counted for radioactivity in a liquid-scintillation counter.

The final aqueous layer obtained thus contained only 0.4% of the [^{14}C]cholate originally present in the assay. The aqueous layer contained 64% of the choloyl-CoA synthesized, the remainder being lost into the butanol phase. The radioactivity of the washed aqueous phase was corrected for the amount of unextracted [^{14}C]cholic acid. The total amount of choloyl-CoA synthesized was obtained by correcting for the amount extracted into the butanol phase. Both corrections were based on multiple experiments in which the amounts of [^{14}C]cholic acid in the water phase and [^{14}C]choloyl-CoA in the butanol phase were measured. Replication of these data from one experiment to another was excellent. None of the detergents studied, except for cetyltrimethylammonium bromide, altered the integrity of the assay system.

The identification of the reaction product as [^{14}C]choloyl-CoA was based on its co-chromatographing with chemically synthesized choloyl-CoA on paper chromatography [Whatman no. 1 in 70% (v/v) propan-2-ol; $R_F = 0.84$] and t.l.c. (silica gel G in chloroform/methanol/water, 75:25:3, by vol.; $R_F = 0.05$). In addition, a compound migrating on thin-layer plates as [^{14}C]cholate was released by boiling in 2 M-NaOH for 30 min.

Gel-filtration chromatography of Triton N-101-solubilized microsomal fraction was conducted on a column (1.8 cm $\times$ 37.5 cm) of Bio-Gel A5m. The column eluent was 50 mM-Tris/HCl (pH 7.4)/0.1 M-NaCl/1% Triton N-101. Microsomal fractions were washed in 0.1 M-NaCl/20 mM-Tris/HCl (pH 7.4)/1% bovine serum albumin, and resuspended in 50 mM-Tris/HCl (pH 7.4)/0.1 M-NaCl containing 2% Triton N-101 at a detergent/microsomal protein ratio of 1:3 (w/w). This suspension was added to the Bio-Gel column, and fractions (2 ml) were collected.

Digestion of microsomal fractions with phospholipase A, purified from *Naja naja* venom, has been described in detail elsewhere (Zakim & Vessey, 1973). Protein was determined by the biuret method after trichloroacetic acid precipitation (Gornall *et al.*, 1949).

Results and Discussion

Effect of pH, microsomal protein and temperature on reaction rate

The effects of pH on the activity of choloyl-CoA synthetase at 30°C are shown in Fig. 1. The optimum

pH is 7.2–7.3. Variation of the concentration of microsomal protein in the assay revealed that the rate of reaction was directly proportional to the concentration of microsomal protein in the assay for concentrations less than 0.8mg of protein/ml. The time-course of the reaction is linear for 5min, but not beyond. Initial rates were measured as a routine between 30 and 60s because there was often a brief, unexplained lag in the rate of the reaction. The rate of reaction was very temperature-dependent. This is reflected by the data in Fig. 2. Also noteworthy is that there are no discontinuities in the Arrhenius-like plot over the temperature range 9–30°C. Discontinuities in such plots have been observed with many membrane-bound enzymes (Vessey & Zakim, 1974). The temperature at which the discontinuity occurs in these cases has been the temperature at which the lipid phase of the membrane undergoes a change of state. The data in Fig. 2 thus suggest that the kinetic properties of choloyl-CoA synthetase are less dependent on enzyme–lipid interactions than are those of many other tightly bound membrane enzymes.

Effect of bivalent cations

We have found that choloyl-CoA synthetase activity has an absolute bivalent-cation requirement when assayed in EDTA-washed microsomal fractions. The previous reports by Bremer (1956) and Elliott (1956) that this activity was stimulated by bivalent cations, but not absolutely dependent on them, can be explained by the fact that the micro-

Fig. 2. Temperature-dependence of the choloyl-CoA synthetase reaction

Initial rates (*v*) were determined by using the standard assay (see under 'Methods') and are expressed as nmol of choloyl-CoA synthesized/min per mg of microsomal protein.

Fig. 1. Effect of pH on choloyl-CoA synthetase activity. Assay mixtures containing 10 μM-[¹⁴C]cholate, 20 mM-ATP, 10 mM-Mg²⁺, 100 mM-Tris/HCl and 0.8 mg of microsomal protein/ml were adjusted to the indicated pH. The reaction was then started by the addition of 100 μM-CoA. Reactions were run for 20, 40 and 60 s at each pH in order to determine the initial rate of reaction. The pH was measured immediately after the assay as a check. Rates are expressed as nmol of choloyl-CoA synthesized/min per mg of microsomal protein.

Table 1. Effect of bivalent cations on choloyl-CoA synthetase

Activity was determined by using the standard assay (see under 'Methods') except that Mg²⁺ was omitted and the indicated additions were made. Metal ions were added at a concentration of 4 mM and EDTA at 1 mM.

Addition	Activity (nmol of choloyl-CoA formed/min per mg of microsomal protein)
—	0.328
EDTA	0
Mg ²⁺	1.80
Mn ²⁺	2.19
Ca ²⁺	0.476
Cu ²⁺	0
Fe ²⁺	0.805
Ba ²⁺	0.307

somal fraction isolated in 0.25M-sucrose contains considerable endogenous bivalent cation, enough in fact to support a low activity (Table 1). As expected, this activity can be abolished by the addition of EDTA (Table 1). Also shown in Table 1 are the

effects of various bivalent cations on the rate of choloyl-CoA synthesis in the presence of endogenous metal. Ba^{2+} is without effect, and Ca^{2+} and Fe^{2+} enhance the rate modestly. The addition of either Mg^{2+} or Mn^{2+} leads to a considerable enhancement of the basal rate. Under the conditions of Table 1, Mn^{2+} gives a higher rate than Mg^{2+} . However, the effect of bivalent cations on the reaction is very complex, and there are conditions (cf. Fig. 3 and discussion below) under which Mg^{2+} gives the same rate as Mn^{2+} . For the purpose of comparing our data with those of Elliott (1956), we have used Mg^{2+} in this study to satisfy the bivalent cation requirement of the enzyme. Under the conditions of the standard assay, the optimum concentration of Mg^{2+} is 4 mM.

Reaction rate as a function of substrate concentration

We were interested to obtain the approximate range of substrate concentrations in which the enzyme would be functioning efficiently. To do this we determined the concentration of a substrate which gives half-maximal rates in the presence of optimum concentrations of the other substrates, i.e. a K_m^{app} (apparent K_m). Variation of the concentration of CoA at optimum concentrations of ATP, cholate and either Mg^{2+} or Mn^{2+} yielded the rate data plotted in Fig. 3 in double-reciprocal form. The extrapolated K_m^{app} for CoA is $50\mu\text{M}$, but in fact the rate of reaction is increased little by concentrations of CoA above $25\mu\text{M}$. A similar type of CoA effect can be seen in the data from a study of the microsomal palmitoyl-CoA synthetase reaction (Pande & Mead, 1968). The data in Fig. 3 also reveal that the complex metal-ion effects may be related to their interaction with CoA.

Variation of the concentration of ATP in the

Fig. 3. Double-reciprocal plot of choloyl-CoA synthetase activity as a function of the concentration of CoA.

The assays contained $16\mu\text{M}$ - $[\text{C}^{14}]$ cholate, 5 mM-ATP and either 5 mM- Mn^{2+} (○) or 5 mM- Mg^{2+} (●). Velocity (v) is expressed as nmol of choloyl-CoA formed/min per mg of microsomal protein.

presence of saturating amounts of CoA, near-saturating concentration of cholate and 4 mM- Mg^{2+} yielded a double-reciprocal plot which was linear up to 3 mM-ATP; ATP becomes inhibitory above 3 mM. The K_m^{app} for ATP obtained from these data was 0.65 mM. The K_m^{app} for cholic acid, estimated from the data in Fig. 4, is $6\mu\text{M}$. This is considerably less than the value of $400\mu\text{M}$ reported by Elliott (1956, 1962). This discrepancy is probably an indirect result of the fact that the assay used by Elliott did not determine true initial rates of the reactions, but the extent of reaction after a 60 min incubation. In addition, Elliott measured product formation indirectly by trapping it with hydroxylamine, which was present in the assay at a very high concentration (0.5 M).

Inhibition studies

Exposure of the microsomal fraction to 1 mM-*N*-ethylmaleimide for 20 min at pH 8.0 and 25°C , followed by removal of reagent by washing with dithiothreitol, caused 80% loss of activity compared with a control incubation; $50\mu\text{M}$ -cholate did not protect.

The data in Table 2 represent a comparison of various conjugated and unconjugated bile acids as inhibitors of $[\text{C}^{14}]$ choloyl-CoA synthesis. Conjugated bile acids are less efficient inhibitors than unconjugated bile acids. Deoxycholate was the most potent inhibitor. The conjugates of both chenodeoxycholate and deoxycholate were better inhibitors than the corresponding cholate conjugates. These inhibitions are consistent with the hypothesis that one enzyme is responsible for the synthesis of the CoA derivatives of all of the major bile acids.

The microsomal fraction from liver also catalyses the synthesis of CoA adducts of medium- and long-chain fatty acids. To investigate the possibility that

Table 2. Effect of conjugated and unconjugated bile acids on choloyl-CoA synthesis

Activity was determined using the standard assay described under 'Methods'. This assay contains $16\mu\text{M}$ -cholate. To the assay was added the indicated bile acid at a concentration of $39\mu\text{M}$.

Addition	Activity (nmol of choloyl-CoA synthesized/min per mg of microsomal protein)
—	1.83
Deoxycholate	0.10
Glycodeoxycholate	1.01
Taurodeoxycholate	1.56
Glycocholate	1.58
Taurocholate	1.69
Chenodeoxycholate	0.29
Taurochenodeoxycholate	1.52
Glycochenodeoxycholate	1.46

cholate might be conjugated via one of these enzymes, we tested butyrate, palmitate and oleate for their ability to act as possible alternate substrate inhibitors of choloyl-CoA synthetase. Butyrate (1mM) had no effect on the activity of choloyl-CoA synthetase; palmitate (0.85mM) inhibited only slightly, and oleate (0.75mM) inhibited by only 50%. Inhibition by oleate was competitive with regard to cholate (Fig. 4). The concentration of cholate in these experiments was 16 μM , about three times the value needed for half-maximal rates at the concentrations of ATP, CoA and Mg^{2+} used. The K_m for palmitate by microsomal palmitoyl-CoA synthetase is approx. 2.8 μM and the K_m for oleate in oleoyl-CoA synthesis is approx. 1.4 μM (Marcel & Suzue, 1972). The concentrations of fatty acids we used were about 100 times K_m values. More extensive inhibition of choloyl-CoA synthetase by palmitate and oleate would be expected, therefore, if these fatty acids and cholate were metabolized by the same acyl-CoA synthetase. It is reasonable to conclude that the synthesis of palmitoyl-CoA, oleoyl-CoA and choloyl-CoA occurs at different active sites. This conclusion is supported by the findings of Siperstein & Murray (1955) that a preparation of palmitoyl-CoA synthetase does not catalyse the synthesis of choloyl-CoA. The data also suggest that inhibition by fatty acid is not important *in vivo* owing to the high concentrations required for inhibition.

Effects of phospholipase A and detergents

A number of studies have demonstrated that the lipid environment of the microsomal membrane

Fig. 4. Double-reciprocal plot of choloyl-CoA synthetase activity as a function of the concentration of cholate in the presence and absence of oleic acid

The assay was the same as the standard assay (see under 'Methods') except that cholate concentration was varied. Rates are expressed as in Fig. 4 for assays containing 0.5 mM-oleic acid (○) or no addition (●).

affects the enzymic properties of some tightly bound membrane enzymes (cf. Vessey & Zakim, 1974; Zakim & Vessey, 1975). We thus studied the effects of modification of the lipid on the activity of the choloyl-CoA synthetase. The lipid phase of the microsomal membrane was modified by treatment with phospholipase A or detergent.

Incubation of microsomal preparations with phospholipase A (*Naja naja*) led to a time-dependent loss of choloyl-CoA synthetase activity, and ultimately to nearly complete loss of activity. Addition of lysophosphatides directly to microsomal fractions had little effect on the activity of choloyl-CoA synthetase (see below), but fatty acids can inhibit (see above). We therefore studied the effect of removing the fatty acids from phospholipase A-treated microsomal preparations by washing with serum albumin. Phospholipase A-treated microsomal preparations were washed in either 0.25M-sucrose or 14% (w/v) albumin/0.12M-sucrose and the final pellet was resuspended in 0.25M-sucrose. Phospholipase A-treated microsomal fractions washed in sucrose retained only 3% of the activity of untreated control microsomal fractions. However, the activity of choloyl-CoA synthetase in phospholipase A-treated microsomal fractions increased from 3 to 82% of the control value after washing in albumin/sucrose. The controls in these experiments were untreated microsomal fractions washed in albumin/sucrose. It appears therefore that treatment of microsomal fractions with phospholipase A does not affect directly the activity of choloyl-CoA synthetase. These data and those for temperature effects indicate that the kinetic properties of choloyl-CoA synthetase are insensitive to changes within its lipid environment.

Further evidence for the general insensitivity of choloyl-CoA synthetase to perturbation of the membrane lipid environment came from studies with detergents. Lysophosphatidylcholine and Triton N-101 have almost no effect on the activity of this enzyme (Fig. 5). Triton X-100 was only slightly inhibitory. Cetyltrimethylammonium bromide shifted the partition coefficient for choloyl-CoA in the water/butanol system in favour of butanol and could not be studied by our assay procedure.

Since cholate is itself a detergent, lack of sensitivity to modification to the lipid milieu may ensure that the conjugation of the bile acids will not be hindered by secondary detergent effects in those instances of high intra-hepatic concentrations of bile salts, as in cholestasis. The above data do not establish, on the other hand, that the normal activity of the enzyme is completely independent of a need for a lipid environment.

The finding that choloyl-CoA synthetase is relatively insensitive to the effects of detergents

Fig. 5. Effect of detergent on choloyl-CoA synthetase activity

To the standard assay containing 0.8mg of microsomal protein/ml (see under 'Methods') was added the indicated amount of the following detergents: egg lysolecithin (○), Triton N-101 (△) or Triton X-100 (□).

suggested that the enzyme might be obtained in soluble form, and we tested this possibility by using Triton N-101. A frozen-thawed microsomal fraction was washed once in 2% serum albumin/50mM-Tris/HCl (pH 7.4)/100mM-NaCl. The resulting pellet was resuspended in 50mM-Tris/HCl (pH 7.4)/100mM-NaCl/2% Triton N-101. The detergent/protein ratio was 1mg of Triton N-101/3mg of microsomal protein. Detergent-treated microsomal fractions (1ml) were placed on a column (1.8cm × 37.5cm) of Bio-Gel A5m prepared and equilibrated with 50mM-Tris/HCl (pH 7.4)/100mM-NaCl/1% Triton N-101. Enzyme activity was eluted as a single band with an approximate mol.wt. of 400000. Activity in these fractions was stable at 4°C. This result means that choloyl-CoA synthetase can be obtained in an active form which is not bound to a membrane. However, this preparation still contains a great deal

of lipid and detergent, so that it is not possible to draw any conclusions with regard to whether or not the enzyme has a lipid requirement.

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